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## Paraoxonase activities and oxidative status during late pregnancy and postpartum period in dairy cattle with and without retained fetal membrane

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**ABSTRACT.** The aim of this study was to evaluate serum paraoxonase 1 (PON1) activities, total antioxidant capacity (TAC), total peroxidation (TPX), oxidative stress index (OSI) and their associations with Retained fetal membrane (RFM) in late pregnancy and postpartum period. Possible relationships have been investigated between these markers and other relevant blood parameters also. Totally 266 pregnant cows were included in this study. Samples were taken in during late pregnancy and postpartum periods. The cows were divided into two groups after giving birth as RFM and Non-Retained fetal membrane (NRFM). The TAC, TPX and OSI values were not different in RFM compared to NRFM in both periods. The PON1 activities of RFM group in both periods were lower than those of the NRFM, however; these variations were not statistically significant. PON1 activities was statistically higher in the late pregnancy both RFM and NRFM groups than postpartum. This observation point out oxidative stress could not relate to pathogenesis of RFM. The PON1 activity was increased physiologically in pregnant cows, and more information is needed to determine whether PON1 may be used to identify cows at high risk of developing RFM. Decreasing serum urea/creatinine ratio, globulin and total protein concentration and increasing albumin/globulin ratio might be a parameter to contributing use in diagnosis of RFM.

**Keywords:** Dairy cattle, Late pregnancy, Paraoxonase, Postpartum, Retained fetal membran

### INTRODUCTION

The retained fetal membrane (RFM) has been defined as a failure to expel the fetal membranes within 8-12 hours after calving (Eiler, 1997; Fourichon et al., 2000). RFM is a complex postpartum syndrome in cows which affects mother and newborn. The etiopathology and biochemical backgrounds of this syndrome are still not clear and require investigation (Kankofer et al., 2005). The incidence of RFM ranges from 4 to 12% of calving. Dystocia, abortions, length of gestation, environmental conditions and imbalances of nutrition are predisposing factors for RFM (Fourichon et al., 2000). RFM can cause

economically as loss of milk, impaired fertility, high culling rate, prenatal mortality, and cost of treatment (Eiler, 1997). The reactive oxygen species are produced continuously by normal metabolic processes, but the rate of production may be increased markedly by various conditions. In many studies have been showed that oxidative stress increased in normal pregnancy. Moreover, literature indicates that increased oxidative stress and decreased antioxidant status are associated with various obstetric pathologies in different mammalian species (Miller et al., 1993; Roche et al., 2000; Gupta et al., 2005a; Gupta et al., 2005b; Castillo et al., 2006; Yokus et al., 2007).

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Several studies have been investigated in blood markers before calving and placenta retention in the cow, and most of these have been targeted dietary indicators such as selenium, vitamin E and lipid peroxidation. Some investigations implicated that supplementation of antioxidant vitamin and element have reduced incidence of retained placenta and improved reproduction of dairy cows (Eger et al., 1985; Campbell and Miller, 1998), contrary to others (Hidiroglou et al., 1987; Stowe et al., 1988).

Paraoxonase 1 (PON1: EC 3.1.8.1) is a calcium-dependent enzyme which synthesized primarily by the liver. PON1 is tightly bound to apolipoprotein A1 of high density lipoprotein (HDL) and circulates as a HDL component in blood of animals. Also it was shown that PON1 protects to oxidation of macromolecules (Juretic et al., 2006). Many of the studies have been implicated that PON1 activity was significantly lower especially in relation to diseases which are characterized with increased oxidative stress (Juretic et al., 2006; Lkeda et al., 1998; Ece et al., 2006). Furthermore, PON1 activities were found to be significantly lower in women with preeclampsia (Kumru et al., 2004; Uzun et al., 2005). Therefore, evaluation of serum PON1 activities may be useful for assessment of RFM in late pregnancy period. However, there is not enough information concerning the activities of PON1 and total anti-oxidant capacity (TAC), total peroxide (TPX), oxidative stress index (OSI) levels in cows with RFM and NRFM. Concentrations of antioxidants can be measure individually in plasma or sera; however measurement of each antioxidant component requires an intensive labor and time. Measuring of TAC levels can provides more biologically relevant information, thus it may be more useful and practical to evaluate the antioxidant status of plasma. In addition to measure of TPX, are in agreement with results by other oxidant stress markers in various disease. Also, OSI measurement has been used in various conditions with increased oxidative stress (Miyazawa, 1989; Erel, 2004).

The knowledge about plasma PON1 activity in veterinary medicine is still not enough (Kankofer et al., 2005, Yokus et al., 2007) and there is limited information concerning the role of the TPX, TAC concentrations and PON activities in RFM during late pregnancy and postpartum. The aim of this study was to investigate possible changes of the TAC, TPX, OSI and PON1 activity in late pregnancy and postpartum periods in healthy cows and to examine the relation with RFM. And also the other blood parameters which are albumin, globulin, total protein, urea, creatinine, HDL were used to identify predictive indicators of risk for RFM in Holstein cows.

## MATERIALS AND METHODS

The study was performed on clinically healthy in 182 Holstein and 84 Holstein crossbred pregnant dairy cows. Blood samples were taken in between the 6-8th month of pregnancy and after partum. If the placenta was not completely expelled in 12 h after calving, it was considered as retained as defined (Parkinson et al., 2001) (group 1) (n=11) and the others were NRFM. In the current study selected in randomly only sixteen cows for control group which was placenta expelled in 12 h (group 2) (n=16).

Blood for PON1, TAC, TPX and biochemical analyses was taken from each cows by jugular venipuncture, using evacuated tubes (Vacutainer®) with or without EDTA, and centrifuged at 1800 x g for 10 minutes. Plasma and sera were stored at -80 °C until analyzed.

TAC levels were determined as described the methods of Erel (2004). According to this method, the hydroxyl radical reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the plasma, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the plasma. The assay results are expressed as mmol Trolox eq./L, and the precision of this assay is lower than rate of 3%.

The TPX concentrations were determined using the FOX2 method Miyazawa (1989) with minor modifications. The FOX2 test system is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides within the plasma samples. In the presence of xylenol orange which produces a colored ferric-xylenol orange complex whose absorbance can be measured. The FOX2 reagent was prepared by dissolving ammonium ferrous sulphate (9.8 mg) in 250 mM H<sub>2</sub>SO<sub>4</sub> (10 ml) to give a final concentration of 250 mM ferrous iron in acid. This solution volume 90 ml was been added to high-performance liquid chromatography (HPLC) grade methanol which containing 79.2 mg butylated hydroxytoluene (BHT). Finally to prepare the reagent was been added 7.6 mg xylenol orange, with stirring (250 mM ammonium ferrous sulphate, 100 mM xylenol orange, 25 mM H<sub>2</sub>SO<sub>4</sub>, and 4 nM BHT, in 90% (v/v) methanol in a final volume of 100 ml). Aliquots (200 mL) of plasma were mixed with 1.8 ml FOX2 reagent. After incubation at room temperature for 30 min, the vials were centrifuged at 12,000 g for 10 min. Absorbance of the supernatant was determined at 560 nm. The total peroxide content of the plasma samples was determined as a difference in absorbance between the

**Table 1.** Comparison of the mean values of various factors in late pregnancy (LP) and post-partum (PP), in cows RFM and NRFM ( $\bar{x}\pm$ SD).

	RFM (n=11)		NRFM (n=16)	
	LP	PP	LP	PP
Urea (mg/dl)	79.5 $\pm$ 30 <sup>3</sup>	55.9 $\pm$ 24 <sup>3</sup>	92.2 $\pm$ 29 <sup>3</sup>	69.7 $\pm$ 58 <sup>3</sup>
Creatinine (mg/dl)	0.64 $\pm$ 0.55	0.6 $\pm$ 0.38	0.6 $\pm$ 0.46	0.55 $\pm$ 0.39
Urea/Creatinine ratio	218 $\pm$ 157 <sup>a,3</sup>	123 $\pm$ 59 <sup>b,3</sup>	251 $\pm$ 175 <sup>a,3</sup>	157 $\pm$ 113 <sup>b,3</sup>
Albumin (g/dl)	3.06 $\pm$ 0.31	3.15 $\pm$ 0.24	3.04 $\pm$ 0.23	3.1 $\pm$ 0.47
Globulin (mg/dl)	3.67 $\pm$ 0.7 <sup>a</sup>	4.88 $\pm$ 0.4	3.82 $\pm$ 0.52 <sup>a</sup>	5.08 $\pm$ 0.51
Albumin/Globulin ratio	0.86 $\pm$ 0.17 <sup>a</sup>	0.65 $\pm$ 0.07	0.81 $\pm$ 0.14 <sup>a</sup>	0.62 $\pm$ 0.12
Total protein (g/dl)	6.74 $\pm$ 0.76 <sup>a,3</sup>	8.04 $\pm$ 0.43 <sup>3</sup>	6.86 $\pm$ 0.44 <sup>a,3</sup>	8.18 $\pm$ 0.64 <sup>3</sup>
TAC (mmol Trolox equiv./l)	1.8 $\pm$ 0.3	1.85 $\pm$ 0.3	1.75 $\pm$ 0.25	1.76 $\pm$ 0.24
TPX ( $\mu$ mol H <sub>2</sub> O <sub>2</sub> /l)	8.3 $\pm$ 1	8.06 $\pm$ 2.31	8.97 $\pm$ 2.43	8.52 $\pm$ 4.39
OSI	0.47 $\pm$ 0.07	0.44 $\pm$ 0.14	0.5 $\pm$ 0.14	0.48 $\pm$ 0.21
HDL-C (mg/dl)	28.5 $\pm$ 12.9	45 $\pm$ 14.9	30.2 $\pm$ 23.8	52.3 $\pm$ 18
PON 1 (U/l)	1641 $\pm$ 353	1297 $\pm$ 422	2231 $\pm$ 502	1448 $\pm$ 330

The measured variables were compared within and between groups.

a, b: when this period was compared with corresponding period in the other group, differences were significant (Independent-Samples t Test) ( $p<0.05$ ). 1, 2, 3: The differences between mean values having same number in each group is significant ( $p<0.05$ ,  $p<0.01$ ,  $p<0.001$  respectively). RFM=Retained fetal membrane. NRFM=Non-Retained fetal membrane. LP=Late Pregnancy. PP= Post-partum. PON1=Paraoxonase 1. TAC=Total antioxidant capacity. TPX=Total peroxidation. OSI=Oxidative stress index. HDL-C=High density lipoprotein.

test and blank samples with using a solution of H<sub>2</sub>O<sub>2</sub> as a standard. The results were expressed as micromoles of H<sub>2</sub>O<sub>2</sub> per liter. The coefficient of variation for individual plasma samples were less than rate of 5%.

To perform the calculation, the result unit of TAC, which mmol Trolox equivalent/l, was converted to mmol Trolox equivalent/l and the OSI value was calculated as formula below;  $OSI = [(Total\ peroxide,\ mmol/L)/(TAC,\ mmol\ Trolox\ equivalent/l)\_100]$ .

Paraoxonase activity was measured according to a method previously described by Furlong et al. (1989). The -contained of assay buffer were 0.132 M Tris HCl (pH 8.5), 1.32 mM CaCl<sub>2</sub> and 2.63 M NaCl. And added to this buffer which freshly prepared paraoxon vol-umed 200 ml of 6 mM (O,O-diethyl-O-p-nitrophenyl-phosphate; Sigma Chemical Co., St. Louis, Mo., USA). The rate of generation at p-nitrophenol was determined using spectrophotometer at 405 nm at 37°C. A molar extinction coefficient of 18.05x10<sup>3</sup> was used for calcu-

lation, with paraoxon as substrate. Paraoxonase activity is expressed as units per liter (one unit is the number of micromoles of paraoxon hydrolysed per minute). Serum concentrations of urea, creatinine, total protein, albumin, globulin, HDL-C were determined using an automated analyzer (Olympus, AU 2700, Germany) with commercially available kits. The coefficients of variation for all variables rates were < 5 %. All data were expressed as mean values with standard deviation ( $\bar{x}\pm$ SD). For the comparison of studied parameters was used paired-samples t test during both periods. The independent-samples t test was performed in order to compare two groups for each evaluated parameters. Pearson's and Spearman's correlation analysis was performed to determine the significance of interactions between various variables in each group. A difference with  $P<0.05$  was considered as a significance. All statistical analyses were made through a software program SPSS version 13.0 (SPSS Inc., Illinois, USA).

## RESULTS

The measured parameters in RFM (group 1) and NRFM (group 2) at late pregnancy and postpartum periods are shown in -1. The results of the analysis were given as mean  $\pm$  standard deviation and showed the determined in both groups and periods in the same table.

The concentrations of TAC, TPX and OSI were not changed in RFM group compared to NRFM group in late pregnancy and postpartum periods. In the cows during postpartum period, the level of PON1 were significantly higher compared to the late pregnancy in both groups ( $p < 0.05$ ). Although, serum PON1 activity of RFM group in both periods were lower than those of the NRFM, whereas there was no any statistically significance. There was a tendency to decrease in TPX and OSI values during late pregnancy in both groups, however; there was no statistically significance. And also levels of plasma TAC was shown no significant decrease during the late pregnancy in RFM groups. The concentrations of urea and creatinine tended to decrease at post partum periods in both groups, but these decreases were not significant as a statistically. The urea/creatinine ratio were decreased during late pregnancy and postpartum in RFM group compared to corresponding periods in NRFM (group 2) ( $p < 0.05$  and  $p < 0.01$  respectively). The concentration of albumin were similar in RFM and NRFM groups ( $p < 0.05$ ). The level of globulin decreased in ( $p < 0.05$ ) the comparison of RFM and NRFM, while albumin/globulin ratio increased ( $p < 0.01$ ) in late pregnancy. Also, concentration of TP was found significantly lower in RFM compared to NRFM in late pregnancy ( $p < 0.05$ ). It was seen that PON activities increased while HDL-C concentrations decreased, however; this correlations was not significant as a statistically ( $r = 0.46$ ,  $p < 0.05$ ). In the current study the incidence of dystocia (4%) was consistent with the Eiler (1997).

## DISCUSSION

Increased oxidative stress has been reported in numerous studies in healthy pregnancy of both animals and human beings (Miller et al., 1993; Roche et al., 2000; Gupta et al., 2005a; Gupta et al., 2005b; Castillo et al., 2006; Yokus et al., 2007). It is suggested that the reason of the increased oxidative stress in pregnant individuals; due to the increased free radicals which caused by increased metabolic activity, (Erel, 2004)

raised insulin resistance (Kocyigit et al., 2004) and negative energy balance (Roche et al., 2000). Another factor for the oxidative stress in pregnancy probably was reduction of an antioxidant reserve in the pregnancy and metabolic adaptation for lactation (Miller et al., 1993).

A few studies assessed the oxidant/anti-oxidant balance using TPX and TAC values in the healthy pregnant cattle. Many investigations suggest that TAC values peaked around the parturition in pregnancy (Miller et al., 1993, Yokus et al., 2007, Brzezinska-Slebodzinska et al., 1994). Furthermore, Castillo *et al.* (2006) were found that TAC values peaked 1 week after calving in parallel with concentrations of malondialdehyde (MDA). Likewise, our previous study, no significant increase in TAC levels and decrease in TPX levels were observed after the parturition as compared to late pregnancy (Yokus et al., 2007). Similarly in this study identify that the plasma TAC levels taken early postpartum period tended to be slightly increased comparing to late pregnancy period in both groups, however; no statistical difference was found. On the other hand, TPX levels in postpartum period tended to decrease compared to the late pregnancy stages in both groups. Although, Castillo et al. (2006) was not evaluated OSI levels, it can be considered that this parameter was not changed because of both increased in levels of lipid peroxidation and TAC. In this respect the results of current study are partly coincide with their studies.

The majority of the human studies were recognized increased oxidative stress in pathological pregnancy (Gupta et al., 2005a; Uzun et al., 2005). These studies have shown plasma TAC activity to be lower in preeclampsia (Harma et al., 2005) and there is an imbalance between lipid peroxidase and antioxidants in preeclampsia (Atamer et al., 2005). Furthermore, OSI values were found to be significantly higher in women with preeclampsia (Davidge et al., 1992).

Etiopathology and the biochemical backgrounds of RFM are still not clear and require investigation (Kankofer et al., 2005). Normally it is expected increased oxidative stress in RFM due to physical effort of calving. Brzezinska et al. (1994) have been reported that there was no difference in the levels of serum vitamin E between cows with RFM and NRFM. Also, Campbell and Miller (1998) suggested that supplementation of vitamin E, did not directly affect the incidence of RFM. These findings are in agreement with this current study. On the contrary, Hidiroglou et al., (1987) found that concentration of plasma vitamin A was significantly lower in cows with RFM compared to NRFM

animals.

While some studies have reported that increased MDA concentrations in buffaloes (Ahmed et al., 2009) and cows with RFM (Gupta et al., 2005b; Kankofer, 2001) the others have reported no significant changes in MDA levels of the cows with RFM (Brzezinska-Slebodzinska et al., 1994; Erişir et al., 2006). Moreover, Kankofer et al. (2005) have shown plasma TAC activity were lower in RFM than in cows NRFM. Thus, earlier studies have shown no consistent for oxidative status associated with RFM. Findings in the current study demonstrate that measured TAC, TPX and OSI levels were not altered in RFM, this observation point out oxidative stress could not contribute in pathogenesis of RFM. This data was confirmed by the results of Brzezinska-Slebodzinska et al. (1994) and Erişir et al. (2006).

Many of the studies have been confirm that PON activity increased in normal pregnancy in humans (Kumru et al., 2004; Uzun et al., 2005). To date, there are no reliable values for PON activities in the veterinary literature, except for a series of studies by Turk et al. (2005) reported that PON activities were decreased early lactation and postpartum periods compared with dry period and late lactation respectively in healthy cattle. Furthermore, our previous study has also shown increased serum PON1 activity in pregnant cattle (Bademkiran et al., 2008). In the present study, the observed increase in PON1 activities at the late pregnancy in both groups are in line with previously published reports. The increased PON1 activity in pregnancy may be results of elevated lipid and lipoprotein metabolism. The results of the present study support of thesis that physiologic variations have to be taken into consideration for the correct interpretation of serum PON1 activities (Kumru et al., 2004; Uzun et al., 2005; Bademkiran et al., 2008).

Several reports suggest that as a result of the nature of its carrier system, serum concentration of PON1 usually are correlated with HDL-C (Juretic, 2006) after the association of PON1 with HDL was first described by Uriel, (1961). On the other hand similar to results of this study, some studies showed that PON1 activities were independent of HDL concentrations (Turk et al., 2005). In this study was seen that PON1 activities increased while HDL-C concentrations decreased, but correlations was not significant as a statistically. The results conflicted probably because of difference of lipid and lipoprotein metabolism in various diseases, race and the physiologic condition (Turk et al., 2005;

Bademkiran et al., 2008).

Many of the studies reported that PON1 activity was significantly lower in pathological pregnancy in humans especially in preeclampsia (Kumru et al., 2004; Uzun et al., 2005). And it is implicated that the reason for decreased PON1 activity in preeclampsia may be depend on the over production of free radicals (Uzun et al., 2005). There is no studies are available to compare to our results on PON1 activity in RFM. According to our results there were decreasing trend in PON1 activities both late pregnancy and postpartum periods in RFM cows but these differences were not significant. These insignificant decreases in PON1 activities at both periods which may be higher demand of antioxidant (Bademkiran et al., 2008).

The elevated levels of serum total protein observed after partum, both RFM and NRFM; reflect increased mobilization of tissue protein in both groups, in accordance with other reports in dairy cattle (Castillo et al., 2006; Yokus et al., 2007). The reason of the decreased in levels of TP and uric acid may be associated with increased requirements of the protein, as a result of transport to the fetus via the placenta in the late pregnancy in both groups. The lower TP concentration in cows with RFM at late pregnancy compared to corresponding period in NRFM cattle ( $p < 0.05$ ) presumably is due to malnutrition.

Lower concentration of urea and urea/creatinine ratio is associated with low protein uptake and high protein requirements. The tendency of the decreased urea and urea/creatinine ratio confirms the increased protein requirements in late pregnancy in both groups. Furthermore, the elevated urea/creatinine ratio is important indicators of the higher glomerular filtration rate that increases especially in late pregnancy due to the increased total blood volume (Yokus et al., 2007).

In conclusion oxidative stress could not relate to pathogenesis of RFM. The PON1 activity can increase physiologically in pregnant cows. The tendency of decreased PON1 activity might be a parameter to contributing use in diagnosis of RFM. However, further studies with larger number of animals are needed to verify these results. The decreasing concentration of serum urea/creatinine ratio, globulin and total protein and increasing albumin/globulin ratio may indicate a risk of placenta retention. ■

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